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GENETIC TOXICITY ASSESSMENT OF TRIAZINE T17-2

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

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Triazine T17-2, a misile gyroscope dampening fluid, was evaluated using *in vitro* bioassays to assess its potential genotoxic activity. The assays conducted were the Ames *Salmonella*/microsomal mutagenicity assay and the Chinese hamster ovary (CHO) gene mutation assay. Triazine did not demonostrate genotoxic potential in either assay therefore the results would predict little or no genetic risk from Triazine T17-2.

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PREFACE

The research reported in this document was conducted by Hazleton Laboratories America, Inc. under a subcontract to NSI Technology Services Corporation in support of the Toxic Hazards Research Unit (THRU). The THRU is the contractor operated effort of the Toxic Hazards Division of the Harry G. Armstrong Aerospace Medical Research Laboratory located at Wright-Patterson Air Force Base, OH. During the initiation and conduct of these studies Lt Col Harvey J. Clewell, III, Lt Col Michael B. Ballinger and Major James McDougal served consecutively as the contract technical monitor.

The experimental work reported here was begun on 16 November 1989 and completed 20 June 1990. The genotoxicity assays were conducted at Hazleton Laboratories America facilities in Kensington, MD. The results of their work were reported to NSI in separate reports on each assay. These separate reports were edited by NSI and organized such that the results of each assay are provided in Sections 3 and 4 of this document. Each paper is authored by the investigator that conducted the study and a discussion paper is provided in Section 2 to collectively consider the results from the individual studies. The final unabridged reports received from Hazleton Laboratories with copies of the raw data, Quality Assurance Statements, and Good Laboratory Practice Compliance and Certification Statements for each of the studies will be archived in the Quality Assurance Archives of the THRU.

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SECTION 1

INTRODUCTION

C.S. Godin

The compound 2,4-Bis(2,4,6,7-tetrachloro-1,1,2,3,3,4,5,5,6,7,7-undecafluoroheptyl)-6-(2-bromo-3-chloro-1,1,2,3,3-pentafluoropropyl)-1,3,5-triazine (Triazine T17-2) is presently being used as a missile gyroscope dampening fluid. The structure of this compound is similar to that of triazine herbicides which are considered mild toxicants. These herbicides require an oral dose of >1000 mg/Kg (Murphy, 1980) or a dermal LD_{50} dose ranging from 5-10 g/Kg (Reinhardt and Brittelli, 1981) to cause an effect and are considered mild skin and eye irritants.

Because the primary route of exposure of Triazine T17-2 would be by dermal absorption or accidental ocular contact, acute eye and skin irriation tests as well as a sensitization study were conducted (Kinkead et al., 1990). The results of this study indicated that Triazine T17-2 was slightly irritating to the conjunctival tissue of rabbit eyes but did not demonstrate an irritation or sensitization hazard.

To our knowledge triazines have not been shown to be genotoxic. However, the genotoxic potential has not been investigated. To investigate the risk of genetic damage as a result of exposure to Triazine T17-2, two mutagenic assays were conducted.

This report is arranged with the results of each assay described in a separate paper (see Sections 3 and 4). Each paper is authored by the investigators that conducted the study and a discussion paper is provided to collectively consider the results from each study (see Section 2).

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Kinkead, E.R., S.K. Bunger and R.W. Wolfe. 1990. Irritation and sensitization studies on Triazine T17-2. AAMRL-TR-90-002. Wright Patterson Air Force Base, OH: Harry G. Armstrong Aerospace Medical Research Laboratory.

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SECTION 2

TRIAZINE T17-2 GENOTOXICITY SUMMARY EVALUATION

B.C. Myhr

Triazine T17-2 was tested for potential genotoxic activity in both bacterial and mammalian cell *in vitro* mutation assay systems, as described below. The substance is a clear, colorless, highly viscous fluid that was found to be soluble in absolute ethanol at concentrations at least as high as 200 mg/ml. Ethanol solutions were prepared just prior to performing the studies, using glass containers and pipets. After dilution into culture medium, solubility was maintained at the highest applied dose of 1000 μg/ml, although precipitation did occur in the Ames test at concentrations exceeding approximately 667 μg per plate.

The Ames *Salmonella* reverse mutation assay was performed with the preincubation method in order to maximize the interaction between the bacteria and Triazine T17-2. Strains TA98, TA100, TA1535, TA1537, and TA1538 were used in the presence and absence of a rat liver S9 metabolic activation system (Aroclor 1254-induced). Triazine T17-2 was not toxic even at the maximum applied concentration of 10,000 μg per plate, with or without the S9 activation system. A dose range of 333 μg per plate to 10,000 μg per plate was assayed for the induction of revertants. No increase in revertants was obtained, which indicated the lack of any mutagenic activity by Triazine T17-2.

In mammalian cell culture, Triazine T17-2 was tested for mutagenic activity at the HGPRT locus in CHO cells. After 4 h exposures in the presence and absence of the rat liver S9 activation system, little or no toxicity was observed up to the highest applied dose of 1000 μg/ml. The mutation assays performed with and without the S9 activation system showed no significant

changes in the mutant frequency over a dose range of 50 μg/ml to 1000 μg/ml. Therefore, Triazine T17-2 was neither toxic nor mutagenic to CHO cells, as detectable at the HGPRT locus.

The results of the above two genetic tests indicate that Triazine T17-2 does not interact with genetic material. Also, no toxicity was observed for very high dose levels. It is unlikely, therefore, that genetic activity would be revealed by other *in vitro* genetic tests, and the results from the two mutation tests would predict essentially no genetic risk from exposure to Triazine T17-2.

SECTION 3

MUTAGENICITY TEST ON TRIAZINE T17-2 IN THE SALMONELLA/REVERSE MUTATION ASSAY (AMES TEST) PREINCUBATION METHOD

T.E. Lawlor and L. Haworth

INTRODUCTION

The mutagenic activity of Triazine T17-2 was examined using the *Salmonella*/Reverse Mutation Assay (Ames Test), Preincubation Method. This assay evaluates the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor-induced rat liver.

The *Salmonella*/Mammalian-microsome reverse mutation assay (Ames Test) detects point mutations, both frameshifts and/or base pair substitutions, in bacteria. The strains of *Salmonella typhimurium* used in this assay are histidine auxotrophs by virtue of conditionally lethal mutations in their histidine operon. When these histidine-dependent cells (*his*-) are exposed to the test article and grown under selective conditions (minimal media with a trace amount of histidine) only those cells which revert to histidine independence (*his*+) are able to form colonies. The trace amount of histidine in the media allows all the plated bacteria to undergo a few cell divisions: this growth is essential for mutagenesis to be fully expressed. The *his*+ revertants are readily discernable as colonies against the limited background growth of the *his*- cells. By utilizing several different tester strains, both base pair substitution mutations and frameshift mutations can be detected. The Ames

Test has been shown to be a sensitive, rapid and accurate indicator of the mutagenic activity of many materials including a wide range of chemical classes.

The tester strains used were TA98, TA100, TA1535, TA1537 and TA1538. In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The *rfa* wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo(a)pyrene) that would otherwise be excluded by a normal intact cell wall. The second mutation, a deletion of the *uvr*B gene, results in a deficient DNA excision repair system which greatly enhances the sensitivity of these strains to some mutagens. Since the *uvr*B deletion extends through the *bio* gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be via a modification of an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strains TA98, TA1537 and TA1538 are reverted from histidine dependence (auxotrophy) to histidine independence (prototropy) by frameshift mutagens. TA1535 is reverted by base substitution mutagens and TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

MATERIALS AND METHODS

The experimental materials, methods and procedures are based on those described by Ames et al (1975) and Yahagi et al (1975).

The test article, Triazine T17-2 (Lot #16-7), a clear, colorless, highly viscous liquid, was stored at room temperature. Ethanol was used as the vehicle and the test article formed a solution at 200 mg per ml which was the most concentrated stock dilution of the test article prepared. The test article remained in solution at all subsequent dilutions prepared for the mutagenicity assay.

Media and Reagents

Top Agar for Selection of Histidine Revertants: Minimal top agar was prepared with 0.7% agar (w/v) and 0.5% NaCl (w/v). After sterilization by autoclaving, the molten top agar was distributed into sterile bottles and stored at room temperature. Immediately before its use in the mutagenicity assay, the top agar was melted and supplemented with 10 ml per 100 ml agar of a sterile solution which contained 0.5 mM L-histidine and 0.5 mM D-biotin.

Minimal Bottom Agar: Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956), supplemented with 0.2% (w/v) glucose.

Nutrient Broth: Nutrient Broth used for growing overnight cultures of the tester strains was Vogel-Bonner salt solution supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

Exogenous Metabolic Activation

Liver Microsomal Enzymes - S9 Homogenate: S9 Liver homogenate for use in the mutagenicity assay, prepared as described below, was purchased commercially from Molecular Toxicology, Inc., College Park, MD, 20742, Lot # 0285, 41.4 mg of protein per ml.

Species, Strain, Sex, Inducer: Liver microsomal enzymes were prepared from male Sprague-Dawley rats that had been injected intraperitoneally with Aroclor 1254 (200 mg per ml in corn oil) at 500 mg/kg, five days prior to preparation of the homogenate. Five days following injection with Aroclor, the rats were sacrificed by decapitation, and their livers were excised.

S9 Characterization: The S9 homogenate was characterized (using the Ames Assay) for its ability to metabolize selected promutagens to their mutagenic forms, as described by deSerres and Shelby (1979).

S9 Mix: The S9 mix was prepared immediately before its use in the mutagenicity assay. One ml of the microsomal enzyme reaction mixture (S9 mix) contained the following components:

H_2O	0.70 ml
1.00M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 ml
0.25M Glucose-6-phosphate	0.02 ml
0.10M NADP	0.04 ml
0.2M $MgCl_2$/0.825M KCl	0.04 ml
S9 Homogenate	0.10 ml

When S9 was required, 0.5 ml of the S9 mix was added to each preincubation mixture.

Sham S9 Mix: The Sham S9 mix was prepared immediately before its use in the mutagenicity assay. One ml of the Sham S9 mix contained the following components:

H_2O	0.90 ml
1.00M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 ml

When S9 was not required, 0.5 ml of the phosphate buffer mix was added to each preincubation mixture.

Test System

Tester Strains: The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535, TA1537 and TA1538. The description of the tester strains that follows is a summarization of the description provided by Ames et al (1975).

TESTER STRAIN GENOTYPES

Histidine Mutation			Additional Mutations		
*his*G46	*his*C3076	*his*D3052	LPS	Repair	R Factor
TA1535	TA1537	TA1538	*rfa*	*uvr*B	-
TA100		TA98	*rfa*	*uvr*B	+R

Source of Tester Strains: The tester strains used were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

Storage of the Tester Strains: Frozen permanent stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing small aliquots (approximately 0.5 - 1.5 ml) at $\leq$-65°C.

Master Plates: Master plates were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine (260 μM), biotin (3 μM), and for strains containing the R-factor, ampicillin (25 μg/ml). Tester strain master plates were stored at 6 ± 4°C.

Preparation of Overnight Cultures: Overnight cultures were prepared by transferring a colony from the appropriate master plate to a flask containing culture medium. In order to assure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring. Inoculated flasks were placed in a shaker/incubator which was programed to begin operation (shaking, 125 ± 25 rpm; incubation, 37 ± 2°C) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began. Cultures were harvested once a predetermined turbidity was reached as determined by a percent transmittance (%T) reading on a spectrophotometer. Overgrowth of cultures can result in their loss of sensitivity to some mutagens. Cultures were removed from incubation when the target %T was reached.

Confirmation of Tester Strain Genotypes: Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay.

rfa Wall Mutation: The presence of the *rfa* wall mutation was confirmed by demonstration of sensitivity to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 μg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

pKM101 Plasmid R-factor: The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic

sensitivity disk containing 10 μg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

Characteristic Number of Spontaneous Revertants: The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 μl aliquots of the culture along with the appropriate vehicle on selective media.

Experimental Design

Mutagenicity Assay: The tester strains used in this study were TA98, TA100, TA1535, TA1537 and TA1538. The assay was conducted using three plates per dose in the presence and absence of microsomal enzymes. Six doses of the test article were tested, from 10,000 to 333 μg per plate in both the presence and absence of S9. (It should be noted that in this report, the doses have been expressed as μg of test article per plate. This reflects the fact that the exposure of the test system to the test article does not cease at the end of the 20 min preincubation preriod. A dose of 10,000 μg per plate indicates that the bacteria are exposed to a concentration of 15,400 μg of test article per ml of the preincubation mixture for 20 minutes prior to being combined with 2 ml of overlay agar and being overlaid onto 25 ml of bottom agar.) Six doses of the test article were tested along with the appropriate vehicle and positive controls. The doses tested were selected based on the results of the dose rangefinding study.

Frequency and Route of Administration: The test system was exposed to the test article via the preincubation modification of the Ames Test originally described by Yahagi et al (1975). This methodology has been

shown to detect mutagenicity with certain classes of chemicals, such as nitrosamines or volatile compounds, which may not be detected in the standard plate incorporation method. All doses of test article, vehicle controls and positive controls were preincubated and plated in triplicate.

Dose Rangefinding Study: The dose rangefinding study was performed using tester strain TA100 both in the presence and absence of microsomal enzymes. Ten doses of test article were tested (one plate per dose). The dose rangefinding study was performed using the same methodology as was used for the mutagenicity assay. Cytotoxicity in this study is detectable as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Routinely, the maximum dose selected to be tested in the mutagenicity assay should demonstrate cytotoxicity if possible.

The growth inhibitory effect (cytotoxicity) of the test article on tester strain TA100 is generally representative of that observed on the other tester strains and because of TA100's comparatively high number of spontaneous revertants per plate, gradations of cytotoxicity can be readily discerned from routine experimental variation. Also, the cytotoxicity induced by a test article in the presence of microsomal enzymes may vary greatly from that observed in the absence of microsomal enzymes. Therefore, this would require that different test article dose ranges be tested in the mutagenicity assay based on the presence or absence of the microsomal enzymes.

Controls

Positive Controls: All combinations of positive controls and tester strains plated concurrently with the assay are listed below.

POSITIVE CONTROL AND TESTER STRAIN COMBINATIONS

Tester Strain	S9 Mix	Positive Control	Conc. per Plate
TA98	+	2-aminoanthracene	2.5 μg
TA98	-	2-nitrofluorene	1.0 μg
TA100	+	2-aminoanthracene	2.5 μg
TA100	-	sodium azide	2.0 μg
TA1535	+	2-aminoanthracene	2.5 μg
TA1535	-	sodium azide	2.0 μg
TA1537	+	2-aminoanthracene	2.5 μg
TA1537	-	ICR-191	2.0 μg
TA1538	+	2-aminoanthracene	2.5 μg
TA1538	-	2-nitrofluorene	1.0 μg

Source and Grade of Positive Control Articles: 2-aminoanthracene, Sigma Chemical Co., practical grade; 2-nitrofluorene, Aldrich Chemical Co., 98%; sodium azide, Sigma Chemical Co., practical grade: ICR-191, Polysciences Inc., >95% pure.

Vehicle Controls: Appropriate vehicle controls were plated for all tester strains both in the presence and absence of S9. The vehicle control was plated, using an aliquot of vehicle equal to the aliquot of test article dilution plated, along with an aliquot of the appropriate tester strain, on selective agar

Sterility Controls: To determine the sterility of the test article, the highest test article dose used in the mutagenicity assay was checked for sterility by plating an aliquot volume equal to that used in the assay on selective agar. To determine the sterility of the S9 mix and of the 0.1M phosphate buffer, a 0.5 ml aliquot of each was plated on selective agar.

Plating Procedures

The plating procedures employed are similar to those described by Ames et al (1975) and Yahagi et al (1975). These prccedures were employed for both the Dose Rangefinding Study and the Mutagenicity Assay.

Test System Identification: Each plate was labeled with a code system which identified the test article, tester strain, test phase, dose and activation condition.

Test Article Plating Procedure: The test article was diluted and the S9 mix was prepared immediately before their use in any experimental procedure.

When S9 mix was required, 0.5 ml of S9 mix was added to 13 x 100 mm glass culture tubes, pre-heated to 37 ± 2°C. To these tubes were added 100 µl of appropriate tester strain and 50 µl of vehicle or test article dilution. When S9 mix was not required, 0.5 ml of 0.1M phosphate buffer was substituted for the S9 mix. After vortexing, the mixture was allowed to incubate for 20 ± 2 minutes at 37 ± 2°C. Two ml of molten selective top agar was added to each tube and the mixture was vortexed and overlaid onto the surface of 25 ml of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay had solidified, the plates were inverted and incubated for 48 ± 8 hours at 37 ± 2°C.

Scoring Plates

Plates which were not scored immediately after the approximately 48 hour incubation period were held at 6 ± 4°C until such time that scoring could occur.

Evaluation of the Bacterial Background Lawn: The condition of the background bacterial lawn was evaluated for evidence of cytotoxicity due to the test article by using a dissecting microscope. The cytotoxicity was

scored relative to the vehicle control plate. In addition to the cytotoxicity, any test article precipitate observed on the plates is also noted at the appropriate dose on the data tables (See Appendix 3-A).

Colony Counting: Revertant colonies for a given tester strain and activation condition were counted either entirely by automated colony counter or entirely by hand. If the plates contained sufficient test article precipitate to interfere with automated colony counting, then they were counted manually.

Analysis of the Data: For all replicate platings, the mean number of revertants per plate was calculated and the standard deviation around the mean was also calculated.

Criteria for Determination of a Valid Test

The following criteria must be met for the assay to be considered valid:

rfa Wall Mutation: In order to demonstrate the presence of the deep rough mutation, all tester strain cultures must exhibit sensitivity to crystal violet.

pKM101 Plasmid R-Factor: In order to demonstrate the presence of the pKM101 Plasmid R-factor, all tester strains must exhibit resistance to ampicillin.

Characteristic Number of Spontaneous Revertants: All tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The acceptable ranges are as follows:

TA98	8 - 60
TA100	60 - 240
TA1535	4 - 45
TA1537	2 - 25
TA1538	3 - 35

Tester Strain Culture Density: In order to ensure that appropriate numbers of bacteria are plated, tester strain culture density must be greater than or equal to 5.0×10^8 bacteria per ml and/or have reached a target level of turbidity demonstrated to produce cultures with a density greater than or equal to 5.0×10^8.

Positive Control Values: All positive controls must exhibit at least a three-fold increase in the number of revertants per plate over the mean value for the vehicle control for the respective strain.

Cytotoxicity: A minimum of three non-toxic doses are required to evaluate assay data.

Evaluation of Test Results

Tester Strains TA98 and TA100: For a test article to be considered positive, it must cause at least a 2-fold increase in the mean revertants per plate of at least one tester strain over the mean vehicle control value for that tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

Tester Strains TA1535, TA1537 and TA1538: For a test article to be considered positive, it must cause at least a 3-fold increase in the mean revertants per plate of at least one tester strain over the mean vehicle control value for that tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

RESULTS AND DISCUSSION

Dose Rangefinding Study

Doses of Triazine T17-2 to be tested in the mutagenicity assay were selected based on the results of the dose rangefinding study conducted on the test article using tester strain TA100 in both the presence and absence of S9 (one plate per dose). Ten doses of test article, from 10,000 to 10.0 μg per plate were tested and the results are presented in Table 3-1. No cytotoxicity was observed in either the presence or absence of S9 as evidenced by a normal background lawn and no decrease was observed in the number of revertants per plate.

Mutation Assay

The results of the dose rangefinding study were used to select 6 doses to be tested in the mutagenicity assay. The doses selected for the mutation assay ranged from 10,000 to 333 μg per plate in both the presence and absence of S9.

The mutagenicity assay results for Triazine T17-2 were acceptable and all criteria for a valid study were met. There were no positive increases in the number of histidine revertants per plate with any of the tester strains either in the presence or absence of S9 (Appendix 3-B).

The results of the *Salmonella*/Reverse Mutation Assay (Ames Test), Preincubation Method, indicate that under the conditions of this study, Triazine T17-2 did not cause a positive increase in the number of histidine revertants per plate of any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor-induced rat liver.

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TABLE 3-1. DOSE RANGEFINDING STUDY

μg/PLATE	TA100 REVERTANTS PER PLATE WITH S9 NUMBER OF COLONIES/PLATE	WITH S9 APPEARANCE OF BACKGROUND LAWN*	WITHOUT S9 NUMBER OF COLONIES/PLATE	WITHOUT S9 APPEARANCE OF BACKGROUND LAWN*
0.00 (Vehicle) (50 μl)	100	1	96	1
Test Article				
10.0	120	1	103	1
33.3	105	1	99	1
66.7	127	1	94	1
100	92	1	95	1
333	113	1	73	1sp
667	113	1	100	1sp
1000	117	1sp	101	1sp
3330	103	1sp	91	1sp
6670	112	1sp	110	1sp
10000	97	1sp	107	2sp

*Background Lawn Evaluation Codes:
1 = normal
2 = slightly reduced
3 = moderately reduced
sp = slight precipitate
mp = moderate precipitate (requires hand count)
4 = extremely reduced
5 = absent
6 = obscured by precipitate
hp = heavy precipitate (requires hand count)

APPENDIX 3-A
BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows:

CODE	DEFINITION	CHARACTERISTICS OF BACKGROUND LAWN
1	Normal	A healthy microcolony lawn.
2	Slightly	A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	A marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
5	Absent	A complete lack of any microcolony lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

SP	Slight Precipitate	Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	The amount of macroscopic precipitate on the plate would interfere with automated counting, thus, requiring the plate to be hand counted.
HP	Heavy Precipitate	The large amount of macroscopic precipitate on the plate makes the required hand counting difficult.

Example: 4-MP would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

Appendix 3-B.
SALMONELLA MUTAGENICITY ASSAY RESULTS
FOR TRIAZINE T17-2
INDIVIDUAL PLATE COUNTS

TEST ARTICLE ID: Triazine T17-2
VEHICLE: Ethanol (100%)
PLATING ALIQUOT: 50 µl

		REVERTANTS PER PLATE															BACKGROUND LAWN*
	DOSE/PLATE	TA98			TA100			TA1535			TA1537			TA1538			
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	
MICROSOMES: Rat Liver																	
VEHICLE CONTROL		30	39	31	135	111	125	18	13	18	10	9	8	17	27	20	1
TEST ARTICLE	333 µg	30	33	27	128	117	115	11	15	24	15	5	15	15	21	21	1
	667 µg	35	26	38	115	117	113	21	17	9	7	7	6	17	17	20	1
	1000 µg	26	35	29	111	111	112	16	13	17	11	5	6	26	26	18	1sp
	3330 µg	37	27	31	107	97	104	22	20	17	5	6	7	23	15	17	1sp
	6670 µg	34	36	33	83	94	120	15	16	20	4	10	7	15	20	24	1sp
	10000 µg	41	28	28	105	114	113	8	16	9	13	8	7	11	21	15	1sp
POSITIVE CONTROL **		917	965	935	1197	835	707	151	195	99	113	118	124	1132	1128	1069	1
MICROSOMES: None																	
VEHICLE CONTROL		29	32	22	83	88	81	17	17	13	10	5	4	18	15	12	1
TEST ARTICLE	333 µg	15	27	35	95	68	79	12	11	13	6	5	4	24	24	25	1
	667 µg	26	20	24	83	76	79	9	13	14	6	5	4	18	20	22	1sp
	1000 µg	26	31	21	89	100	84	15	14	14	4	6	3	16	21	22	1sp
	3330 µg	28	26	15	89	98	76	8	19	12	6	9	5	10	19	18	1sp
	6670 µg	18	27	23	C	92	88	10	11	10	6	8	3	21	23	17	1sp
	10000 µg	19	14	24	94	88	87	9	15	11	7	5	3	22	24	20	1sp
POSITIVE CONTROL ***		284	245	263	758	763	749	581	570	579	1772	2027	2002	526	596	486	1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate
TA1535 2-aminoanthracene 2.5 µg/plate
TA1537 2-aminoanthracene 2.5 µg/plate
TA1538 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate
TA1535 sodium azide 2.0 µg/plate
TA1537 ICR-191 2.0 µg/plate
TA1538 2-nitrofluorene 1.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal
2 = slightly reduced
3 = moderately reduced
4 = extremely reduced
5 = absent
6 = obscured by precipitate
sp = slight precipitate
mp = moderate precipitate (requires hand count)
hp = heavy precipitate (requires hand count)

C = No count due to contamination.

Appendix 3-B cont.

SALMONELLA MUTAGENICITY ASSAY RESULTS FOR
TRIAZINE T17-2
SUMMARY OF TEST RESULTS

TEST ARTICLE ID: Triazine T17-2
VEHICLE: Ethanol (100%)
PLATING ALIQUOT: 50 µl

		MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATIONS										BACKGROUND LAWN*
	DOSE/PLATE	TA98		TA100		TA1535		TA1537		TA1538		
		MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	
MICROSOMES: Rat Liver												
VEHICLE CONTROL		33	5	124	12	16	3	9	1	21	5	1
TEST ARTICLE	333 µg	30	3	120	7	17	7	12	6	19	3	1
	667 µg	33	6	115	2	16	6	7	1	18	2	1
	1000 µg	30	5	111	1	15	2	7	3	23	5	1sp
	3330 µg	32	5	103	5	20	3	6	1	18	4	1sp
	6670 µg	34	2	99	19	17	3	7	3	20	5	1sp
	10000 µg	32	8	111	5	11	4	9	3	16	5	1sp
POSITIVE CONTROL **		939	24	913	254	148	48	118	6	1110	35	1
MICROSOMES: None												
VEHICLE CONTROL		28	5	84	4	16	2	6	3	15	3	1
TEST ARTICLE	333 µg	26	10	81	14	12	1	5	1	24	1	1
	667 µg	23	3	79	4	12	3	5	1	20	2	1sp
	1000 µg	26	5	91	8	14	1	4	2	20	3	1sp
	3330 µg	23	7	88	11	13	6	7	2	16	5	1sp
	6670 µg	23	5	90	3	10	1	6	3	20	3	1sp
	10000 µg	19	5	90	4	12	3	5	2	22	2	1sp
POSITIVE CONTROL ***		264	20	757	7	577	6	1934	141	536	56	1

** TA98 2-aminoanthracene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate
TA1535 2-aminoanthracene 2.5 µg/plate
TA1537 2-aminoanthracene 2.5 µg/plate
TA1538 2-aminoanthracene 2.5 µg/plate

*** TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate
TA1535 sodium azide 2.0 µg/plate
TA1537 ICR-191 2.0 µg/plate
TA1538 2-nitrofluorene 1.0 µg/plate

* Background Lawn Evaluation Codes:
1 = normal
2 = slightly reduced
3 = moderately reduced
4 = extremely reduced
5 = absent
6 = obscured by precipitate
sp = slight precipitate
mp = moderate precipitate (requires hand count)
hp = heavy precipitate (requires hand count)

SECTION 4

MUTAGENICITY TEST ON TRIAZINE T17-2 IN THE CHO/HGPRT FORWARD MUTATION ASSAY

R.R Young

ABSTRACT

The objective of this *in vitro* assay was to evaluate the ability of Triazine T17-2 to induce forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary cells under conditions with and without metabolic activation.

The test material was soluble in ethanol at 100.0 mg/ml. Ethanol was used to prepare primary 100X stocks at lower concentrations. Treatment media were prepared by making 1:100 dilutions of the primary stocks into F12 tissue culture medium. Preliminary cytotoxicity testing showed the test material to be nontoxic to CHO cells under both test conditions.

Mutation assays were performed using both nonactivation and S9 metabolic activation treatment conditions. In each assay, seven test article concentrations were used ranging from 0.05 to 1.0 mg/ml. The test material was not toxic in either mutation assay at any concentration tested. The mutant frequencies of treated cultures varied randomly with dose within the range acceptable for background mutant frequencies. No treated culture had a mutant frequency that was statistically elevated over the mutant frequencies of the vehicle control cultures with the exception of two cultures from the S9 metabolic activation mutation assay. These significant mutant frequencies were within the range of acceptable background mutant frequencies and were consistent with normal assay variation. Therefore, Triazine T17-2 was considered negative for inducing forward mutations at the HGPRT locus in CHO cells under conditions with and without metabolic activation.

INTRODUCTION

Hypoxanthine-guanine phosphoribosyl transferase (HGPRT) is a cellular enzyme that allows cells to salvage hypoxanthine and guanine for use in DNA synthesis. The HGPRT enzyme utilizes the substrates 5-phosphoribosyl-1-pyrophosphate and hypoxanthine or guanine to catalyze the formation of inosine- or guanosine monophosphate. If a purine analog such as 6-thioguanine (TG) is included in the growth medium, the analog will be phosphorylated via the HGPRT pathway and incorporated into nucleic acids, eventually resulting in cellular death. The HGPRT locus is located on the X chromosome. Since only one of the two X chromosomes is functional in the female CHO cells, a single-step forward mutation from HGPRT+ to HGPRT- in the functional X chromosome will render the cell unable to utilize hypoxanthine, guanine, or TG supplied in the culture medium. Such mutants are as viable as wild-type cells in normal medium because DNA synthesis may still proceed by *de novo* synthetic pathways that do not involve hypoxanthine or guanine as intermediates. The basis for the selection of HGPRT- mutants is the loss of their ability to utilize toxic purine analogs (e.g., TG), which enables only the HGPRT- mutants to grow in the presence of TG. Cells which grow to form colonies in the presence of TG are assumed to have mutated, either spontaneously or by the action of the test article, to the HGPRT- genotype.

The objective of this in vitro study was to evaluate the ability of Triazine T17-2 to induce forward mutations at the HGPRT locus in the CHO-K1-BH_4 Chinese hamster ovary (CHO) cell line as assessed by colony growth in the presence of 6-thioguanine (TG). Testing was performed both in the presence and absence of S9 metabolic activation.

MATERIALS AND METHODS

Test Article

The test article Triazine T17-2 (2,4-Bis(2,4,6,7-tetrachloro-1,1,2,3,3,4,5,5,6,7,7-undecafluoroheptyl)-6-(2-bromo-3-chloro-1,1,2,3,3-pentafluoropropyl)-1,3,5-triazine, Lot # 16-7), a clear colorless, viscous liquid, was stored at room temperature.

Indicator Cells

The indicator cells used for this study were Chinese hamster ovary (CHO) cells. The hypodiploid CHO-K1 cell line was originally derived from the ovary of a female Chinese hamster (*Cricetulus griseus*) (Kao and Puck, 1968). Characteristics of the cell line were high clonability (approximately 85%) and rapid doubling time (11-14 h.). The particular clone used in this assay was CHO-K1-BH_4. The BH_4 subclone of CHO-K1 cells, isolated by Dr. A.W. Hsie (Oak Ridge National Laboratory, Oak Ridge, Tennessee), has been demonstrated to be sensitive to many chemical mutagens.

The CHO-K1-BH_4 cells used in this study were obtained in October 1982 from Dr. Hsie. Master stocks of the cells were maintained frozen in liquid nitrogen. Laboratory cultures were maintained as monolayers at 37°C ± 1.5°C in a humidified atmosphere containing 5% ± 1.5% CO_2. Laboratory cultures were periodically checked for karyotype stability and for the absence of mycoplasma contamination. To reduce the negative control frequency (spontaneous frequency) of HGPRT- mutants to as low a level as possible, the cell cultures were exposed to conditions which selected against the HGPRT- phenotype. Cells were maintained in cleansing medium for two to three days, placed in recovery medium for one day and then returned to culture medium. Cleansed cultures were used to initiate mutation assays from three to seven days after having been removed from cleansing medium.

Media

The cells used during experimental studies were maintained in Ham's Nutrient Mixture F12 supplemented with L-glutamine, antibiotics, and fetal bovine serum (8% by volume), hereafter referred to as culture medium. Cleansing medium used for reducing the spontaneous frequency of HGPRT- mutants prior to experimental studies consisted of culture medium (5% serum) supplemented with 5.0×10^{-6} M thymidine, 1.0×10^{-5} M hypoxanthine, 1.0×10^{-4} M glycine, and 3.2×10^{-6} M of either aminopterin or methotrexate. Recovery medium was similar to cleansing medium except that the aminopterin or methotrexate component was removed and the fetal bovine serum was increased to 8% by volume. Selection medium for mutants was hypoxanthine-free F12 medium containing 4 μg/ml (24 mM) of TG and the fetal bovine serum component reduced to 5% by volume.

Control Articles

Negative (media) controls were performed for each portion of the cytotoxicity assay by carrying cells unexposed to the test article through all of the assay operations. In the activation portion of the cytotoxicity assay, the negative control cultures were exposed to the S9 metabolic activation mix. A single negative control culture was used in the cytotoxicity assays. Negative controls were not used in the mutation assays.

The test material was soluble in ethanol. Primary 100X stocks of the test material were prepared using ethanol as the diluent. The primary test material stocks were then diluted 1:100 into culture medium resulting in varying test material concentrations that contained 1% ethanol. Therefore, concurrent vehicle controls were performed for each portion of the assay by exposing cells to 1% ethanol in culture medium for four hours. In the activa-

tion portion of the assays, the vehicle controls were also exposed to the S9 metabolic activation mix. A single culture was used in the cytotoxicity assays and duplicate cultures were used in the mutation assays.

5-Bromo-2'-deoxyuridine (BrdU) is a chemical that is reproducibly and highly mutagenic to CHO-K1-BH_4 cells without S9 metabolic activation. BrdU (Sigma Chemical Co., lot number 81F-0082) was used at a concentration of 50 μg/ml as a concurrent positive control article for nonactivation mutation studies.

3-Methylcholanthrene (MCA) requires metabolic activation by microsomal enzymes to become mutagenic to CHO-K1-BH_4 cells. MCA (Sigma Chemical Co., lot number 70F-0306) was used at 5 μg/ml as a concurrent positive control article for mutation assays performed with S9 activation.

S9 Metabolic Activation System

The *in vitro* metabolic activation system was comprised of rat liver enzymes and an energy producing system, CORE (nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate and an ion mix) prepared in a phosphate buffer. The enzymes were contained in a 9000 x g supernatant (S9 fraction) from liver homogenate prepared from Sprague Dawley rats treated with 500 mg/kg of Aroclor 1254 five days prior to sacrifice (Molecular Toxicology, Inc., lot numbers 0282 and 0292). The treatment with Aroclor 1254 was used to induce mixed function oxidase enzymes capable of transforming chemicals to more active forms.

The preparation of the microsomal fraction was carried out with sterile glassware and solutions at 6° ± 4°C. The livers were excised, weighed, transferred to a vessel containing 3 ml of 0.15M KCl per gram of wet liver weight and homogenized. The homogenate was centrifuged at 9000 x g for 10 minutes. The supernatant was removed and small aliquots distributed into vials which

were immediately frozen. The S9 fraction and reaction mixture (CORE) was retained frozen at about -80°C until used. The S9 and CORE were thawed immediately before use and combined to form the activation system described below.

Component	Final Concentration in Cultures
NADP (sodium salt)	1.0 mM
Glucose-6-phosphate	5.0 mM
Calcium chloride	2.0 mM
Potassium chloride	6.6 mM
Magnesium chloride	2.0 mM
Phosphate	2.0 mM
S9 homogenate	15.0 - 20.0µl/ml

The amount of S9 homogenate per culture depends upon the lot of S9 in use at any time. Before use in the assay, each lot of S9 homogenate was tested. Because the enzymatic activity of S9 homogenate varies among lots, S9 at various concentrations was tested against reference chemicals such as benzo(a)pyrene or 3-methylcholanthrene. The optimum S9 concentration was selected based on induction of HGPRT- mutants in CHO cells, and this amount of S9 was used in all subsequent assays with that particular lot of S9.

Dosing Procedure

The solvent of choice was found to be ethanol. The test material was dissolved at 100.0 mg/ml in ethanol, 100 times the highest desired treatment concentration. Primary 100X test material stock solutions at lower concentrations were then prepared by serial dilution with ethanol. Final 1X dosing stocks were prepared by making 1:100 dilutions of the primary stocks into culture medium containing eight percent fetal calf serum for nonactivation studies and five percent serum for activation studies. The volume of culture medium diluent in the activation studies was reduced to compensate for the volume of S9 reaction mixture used.

Preparations of test material in the vehicle were prepared fresh each day. Treatments were initiated by replacing the culture medium on the cell cultures with the treatment medium containing the test material at the desired concentrations.

Rangefinding Cytotoxicity Testing

After the selection of ethanol as a suitable vehicle, a wide range of test article concentrations was tested for cytotoxicity both with and without S9 metabolic activation. Ten concentrations that spanned a three-log concentration range were used. The applied doses ranged from 0.00195 mg/ml to 1.0 mg/ml. In addition, one negative (media) control and one vehicle control containing 1% ethanol were used in each cytotoxicity assay.

The cells were quantitatively seeded at 200 cells per flask, allowed to attach overnight (16 to 18 h) and exposed to the test or control article for four hours at 37° ± 1.5°C in a humidified atmosphere containing about 5% CO_2. The cells were then washed twice with Dulbecco's phosphate buffered saline (PBS) and incubated in F12 culture medium for six additional days to allow colony development. Colonies were then fixed in alcohol, stained with Giemsa and counted by eye, excluding those with approximately 50 cells or less. Cytotoxicity was expressed as a percentage of colony counts in treated cultures versus control cultures. The preliminary cytotoxicity information was used to select doses for the mutation assay.

Nonactivation Mutagenicity Assay

The assay procedure was based on that reported by Hsie, et al. (1975), and reviewed by Hsie, et al. (1981), with modifications suggested by Myhr and DiPaolo (1978). The cleansed cells were plated at about 3×10^6 cells per 75 cm^2 glass flask on the day before dosing. The time between plating and treatment was about 18 h. Cell cultures were treated with test or control material

for four hours at 37° ± 1.5°C in a humidified atmosphere with about 5% CO_2. Cell cultures normally contain at least 4 x 10^6 cells by the time of treatment termination. After treatment, the cell monolayers were washed twice with phosphate buffered saline, trypsinized, and suspended in culture medium. The cell suspension from each dose level was counted using a Coulter Counter and replated at 1.5 x 10^6 cells into each of two 150-mm dishes and at 200 cells into each of three 60-mm dishes. The small dishes were incubated for seven days to permit colony development and the determination of the cytotoxicity associated with each treatment. The large dishes were incubated for seven days to permit growth and expression of induced mutations. The mass cultures were subcultured every two or three days during the expression period to maintain logarithmic cell growth. At each subculture the cells from the two 150-mm dishes from each dose level were combined and reseeded at about 1.5 x 10^6 cells into each of two 150-mm dishes.

At the end of the expression period (seven days), each culture was reseeded at 2 x 10^5 cells per 100-mm dish (12 dishes total) in mutant selection medium. Also, three 60-mm dishes were seeded at 200 cells each in culture medium to determine the cloning efficiency of each culture. After incubation for seven to ten days, at 37°C ± 1.5°C in a humidified atmosphere with about 5% CO_2, the colonies were fixed with alcohol, stained with Giemsa and counted to determine the number of TG-resistant colonies in mutant selection dishes and the number of colonies in the cloning efficiency dishes. The colonies were counted by eye, excluding those with approximately 50 cells or less.

Activation Mutagenicity Assay

The activation assay was performed independently with its own set of vehicle and positive controls. The procedure was identical to the nonactivation assay except for the addition of the S9 fraction of rat liver homogenate and necessary cofactors during the four-hour treatment period. The fetal bovine serum content of the medium used for dosing was reduced to 5% by volume. The cofactors consisted of nicotinamide adenine dinucleotide phosphate (NADP, sodium salt), glucose-6-phosphate, calcium chloride, potassium chloride, and magnesium chloride, all of which were in a pH 7.8 sodium phosphate buffer.

Data Presentation

The collected data were used to calculate several assay parameters. The chosen combination of raw data and calculated data allows a complete description of events for each treatment condition. The significance of each calculated parameter and its method of calculation are listed below.

Relative Survival to Treatment: This parameter gives the clonal cytotoxicity of each treatment by showing what percentage of the cells were able to form colonies after the treatment period in both the rangefinding cytotoxicity assays and the mutation assays relative to the concurrent vehicle controls. The average number of colonies in three dishes (seeded at 200 cells each) was determined for each treatment condition.

$$\text{Relative Survival (\%)} = \frac{\text{Average no. of colonies per treated culture}}{\text{Average no. of colonies per vehicle control dish}} \times 100\%$$

Relative Population Growth: This parameter shows the cumulative growth of the treated cell population, relative to the vehicle control growth, over the entire expression period and prior to mutant selection. In general, highly toxic treatments will reduce the growth rate as well as the survival.

Values less than 100% indicate growth inhibition. For example, 50% and 25% relative growth values would indicate treated cell populations that were one and two population doublings behind the vehicle control cultures. Treated populations that are more than 2 or 3 doublings behind the control might not achieve maximum expression of the TG-resistant phenotype. The relative population growth is calculated from cell count data not presented in this report and is intended to provide only an approximate indication of growth during the expression period, since cells are easily lost or not completely released by trypsin during the subculture procedures.

$$\text{Relative Population Growth (\%)} = \frac{\text{Treated culture population increase over the expression period}}{\text{Vehicle control population increase over the expression period}} \times 100\%$$

Absolute Cloning Efficiency: The ability of the cells to form colonies at the time of mutant selection is measured by the absolute cloning efficiency (CE). This parameter is used as the best estimate of the cloning efficiency of the mutant cells in the selection dishes. Thus, the observed number of mutant colonies can be converted to the frequency of mutant cells in the treated population.

$$\text{Absolute CE (\%)} = \frac{\text{Average no. of viable colonies per dish}}{200} \times 100\%$$

Mutant Frequency: The mutant frequency is the endpoint of the assay. It is calculated as the ratio of colonies found in thioguanine selection medium to the total number of cells seeded, adjusted by the absolute C.E. The frequency is expressed in units of 10^{-6}, e.g., the number of mutants per one million cells.

$$\text{Mutant Frequency} = \frac{\text{Total mutant clones}}{\text{no. of dishes} \times 2 \times 10^5 \times \text{abs. C.E.}}$$

Assay Acceptance Criteria

An assay normally is considered acceptable for evaluation of the results only if all of the following criteria are satisfied. The activation and non-activation portions of the mutation assay may be performed concurrently, but each portion is, in fact, an independent assay with its own positive and vehicle controls. The activation or nonactivation assays will be repeated independently, as needed, to satisfy the acceptance and evaluation criteria.

1. The average absolute cloning efficiency of the vehicle controls should be between 70% and 115%. A value greater than 100% is possible because of errors in cell counts (usually ± 10%) and dilutions during cloning. Cloning efficiencies below 70% do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies in the range of 50 to 70% and still yield internally consistent and valid results. Assays with cloning efficiencies in this range will be conditionally acceptable and dependent on the scientific judgment of the Study Director. All assays below 50% cloning efficiency will be unacceptable.

2. The background mutant frequency (average of the vehicle controls) is calculated separately for the activation and nonactivation assays, even though the same population of cells may be used for concurrent assays. The activation vehicle controls contain the S9 activation mix and may have a slightly different mutant frequency than the nonactivation vehicle controls. For both conditions, background frequencies for assays performed with different cell stocks are generally 0 to 10 x 10^{-6}. Assays with backgrounds greater than 15 x 10^{-6} will not be used for evaluation of a test article.

3. A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test article clearly shows mutagenic activity as described in the evaluation criteria. If the test article appears to have no or only weak mutagenic activity, an acceptable assay must have a positive control mutant frequency that is significantly elevated over the concurrent vehicle controls ($p \leq 0.01$).

4. For test articles with little or no mutagenic activity, an acceptable assay should include applied concentrations that reduce the clonal survival to approximately 10% to 15% of the average of the vehicle controls, reach the maximum applied concentrations given in the evaluation criteria, reach a concentration that is approximately twice the solubility limit of the test article in culture medium or include a high concentration that is at least 75% of an excessively toxic concentration. There is no maximum toxicity requirement for test articles which clearly show mutagenic activity.

5. Mutant frequencies are normally derived from sets of 12 dishes for the mutant colony count and three dishes for the viable colony count. To allow for contamination losses, an acceptable mutant frequency for treated cultures can be calculated from a minimum of eight mutant selection dishes and two cloning efficiency dishes.

6. The mutant frequencies for five treated cultures are normally determined in each assay. A required number of different concentrations cannot be explicitly stated, although a minimum of three analyzed cultures is considered necessary under the most favorable test conditions in order to accept a single assay for evaluation of the test article.

Assay Evaluation Criteria

Mutation assays are initiated by exposing cell cultures to about six to eight concentrations of test article that are expected, on the basis of preliminary toxicity studies, to span a range of cellular responses from no observed toxicity to about 10% survival. Five dose levels are usually then selected for completion of the mutation assay. These doses should cover a range of toxicities with emphasis placed on the most toxic doses. An assay may need to be repeated with different concentrations to properly evaluate a test article.

The statistical tables provided by Kastenbaum and Bowman (1970) are used to determine whether the results at each dose level are significantly different from the negative controls at 95% or 99% confidence levels. This test compares variables distributed according to Poissonian expectations by summing up the probabilities in the tails of two binomial distributions. The 95% confidence level must be met as one criterion for considering the test article to be active at a particular dose level. In addition, the mutant frequency must meet or exceed 15×10^{-6} in order to compensate for random fluctuations in the 0 to 10×10^{-6} background mutant frequencies that are typical for this assay.

Observation of a mutant frequency that meets the minimum criteria for a positive response in a single treated culture within a range of assayed concentrations is not sufficient evidence to evaluate a test article as a mutagen. The following test results must be obtained to reach this conclusion for either activation or nonactivation conditions:

1. A dose-related or toxicity-related increase in mutant frequency should be observed for at least three doses. However, this depends on the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears. If an increase in mutant frequency is observed for a single

dose near the highest testable toxicity, as defined previously, and the number of mutant colonies is more than twice the value needed to indicate a significant response, the test article generally will be considered mutagenic. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.

2. For some test articles, the correlation between toxicity and applied concentration is poor. The proportion of the applied article that effectively interacts with the cells to cause genetic alterations is not always repeatable or readily controlled. Conversely, measurable changes in the frequency of induced mutants may occur with concentration changes that cause only small changes in observable toxicity. Therefore, either parameter, applied concentration or toxicity (percent survival), can be used to establish whether the mutagenic activity is related to an increase in effective treatment.

3. Treatments that reduce relative clonal survival to less than five percent may be included in the assay but will not be used as sufficient evidence for mutagenicity as it relates to risk assessment.

A test article is evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for a range of applied concentrations that extends to concentrations causing about 10% to 15% survival or extends to a concentration at least 75% of that causing excessive toxicity. If the test article is relatively nontoxic, the maximum applied concentration will normally be 5 mg/ml (or 5 μl/ml) for water-soluble materials or 1 mg/ml (or 1 μl/ml) for materials in organic solvents. If a repeat assay does not confirm an earlier, minimal response as discussed above, the test article is evaluated as nonmutagenic in this assay system.

This presentation may not encompass all test situations, and the Study Director may use other criteria to arrive at a conclusion, especially when data from several repeat assays are available. The interpretation of the results in the Discussion section provides the reasoning involved when departures from the above descriptions occur.

RESULTS AND DISCUSSION

Test Material Handling

The test material, Triazine T17-2, was found to be soluble in ethanol at 100.0 mg/ml. Dilutions were then performed using ethanol to prepare a series of 100X primary stocks at lower concentrations.

Treatment media were prepared by making 1:100 dilutions of the test material stocks into F12 culture medium that contained eight percent fetal bovine serum for the nonactivation studies and five percent serum for the S9 metabolic activation studies. Fresh primary test material stocks were prepared for each experiment. The cells were treated by replacing the media on the cultures with treatment media containing the different concentrations of test or control media.

The test material remained in solution in culture medium from 0.00195 mg/ml to the maximum applied concentration of 1.0 mg/ml. The test material did not alter the pH of the treatment medium outside the range of pH 7.0 to pH 7.8 at any applied concentration.

Rangefinding Cytotoxicity Assay

The sample, Triazine T17-2, was tested in the preliminary rangefinding cytotoxicity assay with and without S9 metabolic activation. Ten test article concentrations were used in each case that ranged from 0.00195 mg/ml to 1.0 mg/ml.

The rangefinding cytotoxicity assay showed that the test material was nontoxic to CHO cells in cultures at all dose levels both with and without S9 metabolic activation (Tables 4-1 and 4-2). The results from the preliminary rangefinding cytotoxicity assays were used to select dose levels for the mutation assays.

Mutation Assay Without Metabolic Activation

Under nonactivation test conditions, Triazine T17-2 showed no dose-related toxicity to CHO cells in culture as measured by either relative clonal survival or relative population growth (Table 4-3). All seven treated cultures survived treatment and were available for analysis.

Without S9 metabolic activation, the mutant frequency of cultures treated with the test material varied within the acceptable range of vehicle control mutant frequency variation which is 0 to 15 x 10^{-6}. There was no positive correlation of mutant frequency with dose and no treated culture had a mutant frequency that was significantly elevated over the average background mutant frequency of the concurrent vehicle controls. Therefore, Triazine T17-2 was evaluated as negative for inducing forward mutations at the HGPRT locus in CHO cells in the absence of S9 metabolic activation.

The positive control treatment with 50 ug/ml 5-bromo-2'-deoxyuridine induced a large, significant ($p \leq 0.01$) increase in mutant frequency. The mutant frequencies of the two vehicle controls were acceptable. Historical control mutant frequency data is presented in Appendix 4-A. The assay results achieved all assay acceptance criteria, which provided confidence in the assumption that the recorded data represented a typical response of the test material in the nonactivation assay system.

Mutation Assay With Metabolic Activation

Under S9 metabolic activation conditions, the cultures treated with Triazine T17-2 showed no dose-related toxicity as measured by either relative clonal survival or relative population growth (Table 4-4). All seven treated cultures survived treatment and were available for analysis. With S9 metabolic activation, the mutant frequency of cultures treated with the test material varied randomly with dose within the acceptable range of vehicle control mutant frequency variation which is 0 to 15 x 10^{-6}. Two of the seven cultures had mutant frequencies that were significantly elevated over the mutant frequencies of the concurrent vehicle control cultures. The significant mutant frequencies were within the range of acceptable background mutant frequencies and were consistent with normal assay variation. Therefore, Triazine T17-2 was evaluated as negative for inducing forward mutations in the presence of S9 metabolic activation.

The positive control treatment with 5 ug/ml 3-methylcholanthrene induced a large, significant ($p \leq 0.01$) increase in mutant frequency which demonstrated the effectiveness of the S9 metabolic activation system and the ability of the test system to detect known mutagens. The mutant frequencies of the vehicle controls were within the acceptable range. The assay results achieved all assay acceptance criteria and provided confidence in the assumption that the recorded data represented typical responses of the test material in the assay system.

CONCLUSION

The test material, Triazine T17-2, is considered negative for inducing forward mutations at the HGPRT locus in Chinese hamster ovary cells under both the S9 metabolic activation and nonactivation conditions of the assay.

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TABLE 4-1. CLONAL CYTOTOXICITY ASSAY WITHOUT METABOLIC ACTIVATION

APPLIED CONCENTRATION MG/ML	NUMBER OF COLONIES	RELATIVE SURVIVAL[a] (PERCENT)	CLONING EFFICIENCY (PERCENT)
NC[b]	184	89.3	
VC, 1%[c]	206	100.0	103.0
TEST MATERIAL			
0.00195	224	108.7	
0.00391	195	94.7	
0.00781	228	110.7	
0.0156	205	99.5	
0.0313	198	96.1	
0.0625	192	93.2	
0.125	187	90.8	
0.25	198	96.1	
0.5	218	105.8	
1.0	218	105.8	

[a] Relative to negative control
[b] NC = NEGATIVE CONTROL, F12 MEDIUM
[c] VC = VEHICLE CONTROL, 1% ETHANOL

TABLE 4-2. CLONAL CYTOTOXICITY ASSAY WITH METABOLIC ACTIVATION

APPLIED CONCENTRATION MG/ML	NUMBER OF COLONIES	RELATIVE SURVIVAL[a] (PERCENT)	CLONING EFFICIENCY (PERCENT)
NC[b]	123	93.2	
VC, 1%[c]	132	100.0	66.0
TEST MATERIAL			
0.00195	120	90.9	
0.00391	106	80.3	
0.00781	138	104.5	
0.0156	100	75.8	
0.0313	157	118.9	
0.0625	166	125.8	
0.125	154	116.7	
0.25	146	110.6	
0.5	129	97.7	
1.0	113	85.6	

[a] RELATIVE TO VEHICLE CONTROL
[b] NC = NEGATIVE CONTROL, F12 MEDIUM
[c] VC = VEHICLE CONTROL, 1% ETHANOL

TABLE 4-3. MUTATION ASSAY WITHOUT METABOLIC ACTIVATION

NONACTIVATION TEST CONDITION	SURVIVAL TO TREATMENT % VEH. CONTROL	RELATIVE POPULATION GROWTH (% OF CONTROL)	TOTAL MUTANT COLONIES	ABSOLUTE C.E. ± S.D (%)	MUTANT FREQ IN 10^{-6} UNITS
Vehicle Control[b]	110.4	107.5	23	103.9 ± 7.3	9.2
Vehicle Control	89.6	92.5	18	99.9 ± 4.2	7.5
Positive Contrcl (50 µg/ml BrdU)[c]	62.2	41.9	333	104.5 ± 8.3	132.8[d]
TEST ARTICLE					
0.05 mg/ml	88.0	76.9	4	108.5 ± 6.9	1.5
0.1 mg/ml	113.2	81.5	20	116.9 ± 6.6	7.1
0.2 mg/ml	84.5	80.7	19	115.9 ± 9.8	6.8
0.4 mg/ml	80.6	89.7	18	106.7 ± 2.3	7.0
0.6 mg/ml	85.0	86.7	16	102.5 ± 5.9	6.5
0.8 mg/ml	83.0	91.7	8	105.5 ± 9.1	3.2
1.0 mg/ml	91.3	97.5	16	99.0 ± 8.3	6.7

[a]Mutant Frequency = Total mutant colonies/(No. of dishes x 2 x 10^5 x absolute C.E.)
[b]Vehicle control = 1% Ethanol
[c]BrdU = 5-Bromo-2'-deoxyuridine
[d]Significant increase: Kastenbaum Bowman test $p \leq 0.01$ and mutant frequency $\geq 15 \times 10^{-6}$.

TABLE 4-4. MUTATION ASSAY WITH METABOLIC ACTIVATION

ACTIVATION TEST CONDITION	SURVIVAL TO TREATMENT % VEHICLE CONTROL	RELATIVE POPULATION GROWTH (% OF CONTROL)	TOTAL MUTANT COLONIES	ABSOLUTE C.E. ± S.D. %	MUTANT FREQ IN 10^{-6} UNITS
Vehicle Control[b]	102.5	98.8	4	101.4 ± 7.7	1.6
Vehicle Control	97.5	101.2	7	100.5 ± 4.6	2.9
Positive Control (5 µg/ml 3-MCA)[c]	98.0	62.6	843	97.4 ± 9.6	360.6[d]
TEST ARTICLE					
0.05 mg/ml	95.8	89.7	11	112.2 ± 4.7	4.1
0.1 mg/ml	100.1	94.7	7	98.0 ± 4.0	3.0
0.2 mg/ml	92.9	104.0	6	89.0 ± 10.5	2.8
0.4 mg/ml	81.9	96.7	24	98.4 ± 4.8	10.2[e]
0.6 mg/ml	84.0	110.8	11	104.5 ± 4.8	4.4
0.8 mg/ml	101.8	95.2	20	94.0 ± 4.5	8.9[e]
1.0 mg/ml	102.0	81.0	6	101.4 ± 8.2	2.5

[a]Mutant Frequency = Total mutant colonies/(No. of dishes x 2 x 10^5 x absolute C.E.)
[b]Vehicle control = 1% Ethanol
[c]3-MCA = 3-Methylcholanthrene
[d]Significant increase: Kastenbaum Bowman test $p \leq 0.01$ and mutant frequency $\geq 15 \times 10^{-6}$.
[e]Kastenbaum Bowman test $p \leq 0.01$ but mutant frequency is within acceptable background range ($< 15 \times 10^{-6}$).

APPENDIX 4-A HISTORICAL CHO HGPRT ASSAY MUTANT FREQUENCY CONTROL DATA

A. Nonactivation Studies

1. Pooled negative and solvent controls

Mean (± SD)	$3.9 \pm 2.9 \times 10^{-6}$
Range	0 to 16.8×10^{-6}
Number of experiments	50
Number of controls	88

2. Positive controls (50 μg/ml 5-bromo-2'deoxyuridine)

Mean (± SD)	$121.6 \pm 27.9 \times 10^{-6}$
Range	38.7 to 165.6×10^{-6}
Number of experiments	50
Number of controls	59

B. Activation Studies

1. Pooled negative and solvent controls

Mean (± SD)	$2.9 \pm 2.1 \times 10^{-6}$
Range	0 to 10.0×10^{-6}
Number of experiments	50
Number of controls	86

2. Positive controls (5 μg/ml 3-methylcholanthrene)

Mean (± SD)	$370.0 \pm 173.3 \times 10^{-6}$
Range	152.3 to 941.6×10^{-6}
Number of experiments	50
Number of controls	61

The historical control data was compiled from the most recent fifty experiments. Because some experiments contained duplicate controls, the number of independent control cultures exceeded the number of experiments.